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(57) Abstract

A method for inhibiting the rejection of transplanted grafts is disclosed. The method comprising administering an effective amount of an antagonist of CCR1 function to a graft recipient. Also disclosed is a method of inhibiting ischemia/reperfusion injury comprising administering to a subject in need thereof an effective amount of an antagonist of CCR1 function. The disclosed methods can also comprise the co-administration of one or more additional therapeutic agents, for example, immunosuppressive agents and cell adhesion inhibitors.

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METHODS FOR PREVENTING GRAFT REJECTION AND ISCHEMIA-REPERFUSION INJURY

RELATED APPLICATION

This application is a continuation-in-part of U. S. Serial No. 09/239,283, filed January 29, 1999, the entire teachings of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

In many instances, the best and only treatment available to patients suffering from certain end stage degenerative conditions or congenital genetic disorders is transplantation of a healthy graft (e.g., organs, tissues). Advances in surgical techniques and post-operative immunosuppressive therapy have mitigated some of the barriers to long-term survival of grafts and graft recipients, and ushered this once experimental therapy into wider clinical practice.

A major barrier to the long-term survival of transplanted grafts is rejection by the recipient's immune system. Graft rejection can be classified as hyper-acute rejection which is mediated by preformed antibodies that can bind to the graft and are present in the circulation of the recipient, acute rejection which is mediated by the recipient's cellular immune response or chronic rejection which occurs via a multi-factorial process that includes an immune component. The practice of matching the allelic variants of cellular antigens, most notably major histocompatability antigens (MHC), also referred to as tissue typing, as well as matching of the blood type of the donor and recipient has reduced the incidence of hyper-acute rejection. However, most grafts which are transplanted do not exactly match the tissue type of the recipient (e.g., allografts) and will not remain viable without therapeutic intervention.

The rejection of allografts can be inhibited by long-term (e.g., life-long) prophylactic immunosuppressive therapy, most notably with agents that inhibit calcineurin (e.g., cyclosporin A (CsA), FK-506). Immunosuppressive therapy not

only inhibits rejection of the graft, but can render the recipient susceptible to infection with, for example, viruses, bacteria and fungi (e.g., yeasts, molds), and at higher risk for the development of certain malignancies. Additionally, therapeutic doses of immunosuppressive agents can produce adverse side effects, such as diabetes mellitus, neurotoxicity, nephrotoxicity, hyperlipidemia, hypertension, hirsutism and gingival hyperplasia (Spencer, C.M., *et al.*, *Drugs 54*(6):925-975 (1997)). Thus, the degree of immunosuppression must be carefully tailored to prevent rejection of the graft and to preserve the general health of the recipient.

Despite such prophylactic immunosuppression, the acute and chronic rejection of grafts remains a clinical problem. Acute episodes of rejection are characterized by infiltration of the graft by the recipient's leukocytes (e.g., monocytes, macrophages, T cells) and cellular necrosis. These episodes usually occur during the days to months following transplantation. Acute rejection has been treated with high doses of certain immunosuppressive agents, such as, glucocorticoids (e.g., prednisone) and certain antibodies which bind to leukocytes (e.g., OKT3). However, these therapies do not always stop the rejection, are associated with systemic side effects and can lose efficacy in cases of recurrent rejection activity.

Chronic rejection becomes the major cause of graft failure and recipient death for those patients that survive past the first year. Evidence of chronic rejection can be found in about 40-50% of heart and/or lung allograft recipients who survive for five years, and most kidney grafts succumb to chronic rejection. The pathogenesis of chronic rejection is complex and involves accelerated arteriosclerosis (e.g., atherosclerosis) of the graft-associated vasculature and leukocyte infiltration. Unlike acute rejection episodes, chronic rejection is not generally responsive to further immunosuppressive therapy. Furthermore, the graft accelerated arteriosclerosis characteristic of chronic rejection is generally diffuse and not amenable to conventional therapeutic procedures (e.g., angioplasty, bypass grafting, endarterectomy). Thus, patients who chronically reject their grafts can require a second transplant. (Schroeder J.S. "Cardiac Transplantation", pp. 1298-1300; Maurer, J.R. "Lung Transplantation", pp. 1491-1493; Carpenter, C.B. and Lazarus, J.M. "Dialysis and Transplantation in the Treatment of Renal Failure", pp.

1524-1529; Dienstag, J. "Liver Transplantation", pp. 1721-1725; all in *Harrison's Principles of Internal Medicine*, 14th ed., Fauci et al. Eds. McGraw Hill (1998)).

The flow of oxygenated blood to all transplanted grafts is stopped during graft procurement and transplantation surgery. The lack of blood results in hypoxia and necrosis of some or all of the cells of the graft, depending on the amount of time that blood flow is stopped. The resulting damage or injury is apparent after the flow of blood is returned (reperfusion). This type of injury, referred to as ischemia/reperfusion injury, can lead to the death of endothelial cells which line the graft associated blood vessels (e.g., arteries) (Gohra, H, *et al.*, *Transplantation, 60*(1):96-102 (1995)). Thus, ischemia/reperfusion injury can be a contributing factor to graft accelerated arteriosclerosis and graft rejection.

A need exists for therapeutic methods for preventing graft rejection and ischemia/reperfusion injury.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a Kaplan-Meier survival curve that illustrates the survival of CCR1-/- mice (-◇-) and wild type B6/129 mice (-△-) following renal ischemia/reperfusion. CCR1 -/- mice survived significantly longer than B6/129 mice ($p<0.001$, log rank sum-test, survival limited to 100 days).

Figure 2 is a histogram illustrating the serum creatinine levels of CCR1-/- and B6/129 mice following renal ischemia/reperfusion or sham surgery (unilateral nephrectomy without ischemia/reperfusion). Creatinine levels were measured 2, 7 and 100 days following ischemia/reperfusion or sham surgery. Only B6/129 (control) mice had significantly elevated serum creatinine levels 2 and 7 days following ischemia/reperfusion (**$p<0.005$). All groups had elevated levels of serum creatinine 100 days after ischemia/reperfusion or sham surgery (*$p<0.01$).

Figure 3 is a histogram illustrating serum alanine aminotransferase (SGPT) levels, an indicator of liver dysfunction, in CCR1-/- (KO) and B6/129 (WT) mice at predetermined times following hepatic ischemia/reperfusion. Also shown are the SGPT levels in the serum of B6/129 mice that underwent surgery but did not experience hepatic ischemia/reperfusion (sham). CCR1-/- mice experienced significantly less liver dysfunction than B6/129 mice, as assessed by serum SGPT

levels, over the period from 0.5 to 7 days following hepatic ischemia/reperfusion (*$p<0.0001$).

Figure 4A is a histogram illustrating concanavilin-A-induced proliferation of T cells in cultures of splenocytes isolated from CCR1-/- or B6/129 mice (CCR1+/+). Concanavilin-A-induced T cell proliferative responses in cultures of cells from CCR1-/- mice or cultures of B6/129 mice were about equivalent. Data are mean ± standard deviation of six cultures that were stimulated for 48 hours. The presented data are representative of four assays.

Figure 4B is a histogram illustrating mixed lymphocyte responses (MLR) of cells isolated from CCR1-/- mice (CCR1 KO) or wild type B6/129 mice (WT) stimulated with allogeneic splenocytes (isolated from Balb/c mice). Cells from both CCR1-/- and wild type B6/129 mice displayed robust MLRs toward mitomycin-c treated Balb/c stimulator cells (**$p<0.001$ versus unstimulated CCR1-/- or unstimulated wild type B6/129 responder cells). CCR1-/- cells proliferated less than wild type cells in the assay (*$p<0.01$). Data are mean ± standard deviation of six 5-day cultures. The presented data are representative of four assays. BALB/c stim.: mitomycin-c treated splenocytes isolated from a Balb/c mouse; WT resp.: splenocytes isolated from B6/129 mouse; CCR1 KO resp.: splenocytes isolated from CCR1-/- mouse; BALB/c→WT: splenocytes isolated from B6/129 mouse stimulated with mitomycin-c treated splenocytes isolated from a Balb/c mouse; BALB/c→KO: splenocytes isolated from CCR1-/- mouse stimulated with mitomycin-c treated splenocytes isolated from a Balb/c mouse.

SUMMARY OF THE INVENTION

The invention relates to transplantation and to promoting the viability of transplanted grafts. In one aspect, the invention relates to a method for inhibiting (reducing or preventing) graft rejection (e.g., acute rejection, chronic rejection). In one embodiment, the method comprises administering to a graft recipient an effective amount of an antagonist of CCR1 function. In another embodiment, the graft is an allograft. In a particular embodiment, the allograft is a heart. In a preferred embodiment, the method comprises administration of an antagonist of CCR1 function and one or more immunosuppressive agents to a graft recipient.

In another aspect, the invention relates to a method for inhibiting (reducing or preventing) ischemia/reperfusion injury. In one embodiment, the method comprises administering to a subject in need there of an effective amount of an antagonist of CCR1 function. In some embodiments, the ischemia/reperfusion injury can be a consequence of trauma or a medical procedure, for example, surgery. In other embodiments, the ischemia/reperfusion injury can be the result of a pathological condition, for example, arteriosclerosis, myocardial infarction, stroke or transient ischemic attack. In a particular embodiment, the ischemia/reperfusion injury is a consequence of graft transplantation. In another particular embodiment, the graft is a kidney. In another embodiment, the method comprises administration of an antagonist of CCR1 function and one or more additional therapeutic agents, for example, thrombolytic agents, cell adhesion inhibitors, anti-coagulants, anti-thrombotic agents and activators or inhibitors of nitric oxide synthase.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to transplantation and the viability of transplanted grafts. Specifically, the invention relates to inhibiting graft rejection (e.g., acute graft rejection, chronic graft rejection) by administering to a graft recipient an effective amount of an antagonist of the mammalian (e.g., human) C-C chemokine receptor, CCR1.

Chemokines are a family of proinflammatory mediators that promote recruitment and activation of multiple lineages of leukocytes (e.g., lymphocytes, macrophages). They can be released by many kinds of tissue cells after activation. Continuous release of chemokines at sites of inflammation can mediate the ongoing migration and recruitment of effector cells to sites of chronic inflammation. The chemokines are related in primary structure and share four conserved cysteines, which form disulfide bonds. Based upon this conserved cysteine motif, the family can be divided into distinct branches, including the C-X-C chemokines (α-chemokines), and the C-C chemokines (β-chemokines), in which the first two conserved cysteines are separated by an intervening residue, or are adjacent residues, respectively (Baggiolini, M. and Dahinden, C. A., *Immunology Today*, *15*:127-133 (1994)).

The C-X-C chemokines include a number of potent chemoattractants and activators of neutrophils, such as interleukin 8 (IL-8), PF4 and neutrophil-activating peptide-2 (NAP-2). The C-C chemokines include, for example, RANTES (Regulated on Activation, Normal T Expressed and Secreted), the macrophage inflammatory proteins 1α and 1β (MIP-1α and MIP-1β), eotaxin and human monocyte chemotactic proteins 1-3 (MCP-1, MCP-2, MCP-3), which have been characterized as chemoattractants and activators of monocytes or lymphocytes. Chemokines, such as RANTES and MIP-1α, have been implicated in human acute and chronic inflammatory diseases including respiratory diseases, such as asthma and allergic disorders.

The chemokine receptors are members of a superfamily of G protein-coupled receptors (GPCR) which share structural features that reflect a common mechanism of action of signal transduction (Gerard, C. and Gerard, N.P., *Annu Rev. Immunol., 12:*775-808 (1994); Gerard, C. and Gerard, N. P., *Curr. Opin. Immunol., 6*:140-145 (1994)). Conserved features include seven hydrophobic domains spanning the plasma membrane, which are connected by hydrophilic extracellular and intracellular loops. The majority of the primary sequence homology occurs in the hydrophobic transmembrane regions with the hydrophilic regions being more diverse. The receptors for the C-C chemokines include: CCR1 which can bind, for example, MIP-1α, RANTES, MCP-2, MCP-3, MCP-4, CKbeta8, CKbeta8-1, leukotactin-1, HCC-1 and MPIF-1; CCR2 which can bind, for example, MCP-1, MCP-2, MCP-3 and MCP-4; CCR3 which can bind, for example, eotaxin, eotaxin-2, RANTES, MCP-2, MCP-3 and MCP-4; CCR4 which can bind, for example, TARC, RANTES, MIP-1α and MCP-1; CCR5 which can bind, for example, MIP-1α, RANTES, and MIP-1β; CCR6 which can bind, for example, LARC/MIP-3α/exodus; CCR7 which can bind, for example, ELC/MIP-3β and CCR8 which can bind, for example I-309 (Baggiolini, M., *Nature 392*:565-568 (1998); Luster, A.D., *New England Journal of Medicine, 338*(7):436-445 (1998); Tsou, *et al., J. Exp. Med., 188*:603-608 (1998); Nardelli, *et al., J Immunol, 162*(1):435-444 (1999); Youn, *et al., Blood, 91*(9):3118-3126 (1998); Youn, *et al., J Immunol, 159*(11):5201-5201 (1997)).

CCR1 as well as processes and cellular responses mediated by CCR1, are involved in rejection of transplanted grafts. As described herein, studies of allograft survival using a murine cardiac transplantation model were undertaken. Mice which lack functional chemokine receptor CCR1 as a result of targeted disruption of the CCR1 gene (CCR1 KO; Gerard, C. *et al.*, *J. Clin. Invest. 100*:2022-2027 (1997)) did not reject transplanted allografts, which were mismatched at MHC class I and MHC class II, as rapidly as control mice which comprise a functional CCR1 gene and are otherwise genetically identical to CCR1 KO mice (Example 1, Table 1, groups 1 and 2).

As described herein, administration of low dose immunosuppressive therapy (cyclosporin A) to CCR1 +/+ control mice resulted in only a 2-3 day increase in graft viability as compared with untreated CCR1 +/+ animals (Example 1, Table 1, group 3). Surprisingly, administration of the same low dose of CsA with inhibition of CCR1 function led to permanent (>100 days) engraftment in CCR1 KO mice which received CsA for a maximum period of only 21 days (Table 1, group 4).

In further studies, allografts that were mismatched only at MHC class II were transplanted into CCR1 KO and CCR1+/+ control mice. As expected, the partial tissue match led to prolonged survival of the graft in CCR1+/+ control mice. However, all CCR1+/+ control mice still rejected the graft by the thirty-fifth day (Example 1, Table 1, group 5). Partial matching of the MHC antigens, like low dose immunosuppression, with inhibition of CCR1 function led to permanent (>100 days) engraftment (Example 1, Table 1, group 6).

Histological examination of permanently engrafted hearts removed from group 4 and group 6 (see Table 1) mice at 100 days after transplantation revealed only minimal mononuclear cell infiltration and no evidence of transplant accelerated arteriosclerosis.

The survival of Class I and Class II mismatched allografts can be prolonged by the administration of anti-CD4 monoclonal antibody (mAb) (Mottram, *et al.*, *Transplantation, 59*:559-565 (1995)). However, the long-term survival of these grafts is complicated by the development of chronic rejection with wide-spread arteriosclerosis in the vasculature of the graft (Hancock, *et al.*, *Nature Medicine, 4*:1392-1396 (1998)).

As described herein, Class I and Class II mismatched allografts survived for 60 days in CCR1 KO and CCR1 +/+ control mice that received anti-CD4 mAb therapy. Morphological examination of grafts removed from CCR1 +/+ control recipients at sixty days revealed severe arteriosclerosis. In contrast, grafts removed from CCR1 KO recipients showed no evidence of arteriosclerosis (Example 2, Table 2). Thus, disruption of CCR1 function can provide the dual benefit of inhibiting both acute and chronic rejection of allografts.

Accordingly, a first aspect of the invention provides a method for inhibiting rejection (e.g., acute and/or chronic rejection) of a graft, comprising administering to a graft recipient an effective amount of an antagonist of CCR1 function.

CCR1 antagonists

As used herein, the term "antagonist of CCR1 function" refers to an agent (e.g., a molecule, a compound) which can inhibit a (i.e., one or more) function of CCR1. For example, an antagonist of CCR1 function can inhibit the binding of one or more ligands (e.g., MIP-1α, RANTES, MCP-2, MCP-3, MCP-4, CKbeta8, CKbeta8-1, leukotactin-1, HCC-1, MPIF-1) to CCR1 and/or inhibit signal transduction mediated through CCR1 (e.g., GDP/GTP exchange by CCR1 associated G proteins, intracellular calcium flux). Accordingly, CCR1-mediated processes and cellular responses (e.g., proliferation, migration, chemotactic responses, secretion or degranulation) can be inhibited with an antagonist of CCR1 function.

Preferably, the antagonist of CCR1 function is a compound which is, for example, a small organic molecule, natural product, protein (e.g., antibody, chemokine, cytokine), peptide or peptidomimetic. Several molecules that can antagonize one or more functions of chemokine receptors (e.g., CCR1) are known in the art, including the small organic molecules disclosed in, for example, international patent application WO 97/24325 by Takeda Chemical Industries, Ltd.; WO 98/38167 by Pfizer, Inc.; WO 97/44329 by Teijin Limited; WO 98/04554 by Banyu Pharmaceutical Co., Ltd.; WO 98/27815, WO 98/25604, WO 98/25605, WO 98/25617 and WO 98/31364 by Merck & Co., Inc.; WO 98/02151 and WO 99/37617, by LeukoSite, Inc.; WO 99/37651 and WO 99/37619 by LeukoSite, Inc.,

et al.; United States Provisional Patent Application Number 60/021,716, filed July 12, 1996; United States Patent Application Numbers: 09/146,827 and 09/148,236, filed September 4, 1998; Hesselgesser *et al., J. Biol. Chem. 273*(25):15687-15692 (1998); and Howard *et al., J. Medicinal Chem. 41*(13):2184-2193 (1998); proteins, such as antibodies (e.g., polyclonal sera, monoclonal, chimeric, humanized) and antigen-binding fragments thereof (e.g., Fab, Fab', $F(ab')_2$, Fv), for example, those disclosed in Su *et al., J. Leukocyte Biol. 60*:658-656 (1996); chemokine mutants and analogues, for example, those disclosed in U.S. Patent No. 5,739,103 issued to Rollins *et al.*, WO 96/38559 by Dana Farber Cancer Institute and WO 98/06751 by Research Corporation Technologies, Inc.; peptides, for example, those disclosed in WO 98/09642 by The United States of America. The entire teachings of each of the above cited patent applications and references is incorporated herein by reference.

Antagonists of CCR1 function can be identified, for example, by screening libraries or collections of molecules, such as, the Chemical Repository of the National Cancer Institute (U.S.A.), as described herein or using other suitable methods.

Another source of antagonists of CCR1 function are combinatorial libraries which can comprise many structurally distinct molecular species. Combinatorial libraries can be used to identify lead compounds or to optimize a previously identified lead. Such libraries can be manufactured by well-known methods of combinatorial chemistry and screened by suitable methods, such as the methods described herein.

The term "natural product", as used herein, refers to a compound which can be found in nature, for example, naturally occurring metabolites of marine organisms (e.g., tunicates, algae) and plants and which possess biological activity, e.g., can antagonize CCR1 function. For example, lactacystin, paclitaxel and cyclosporin A are natural products which can be used as anti-proliferative or immunosuppressive agents.

Natural products can be isolated and identified by suitable means. For example, a suitable biological source (e.g., vegetation) can be homogenized (e.g., by grinding) in a suitable buffer and clarified by centrifugation, thereby producing an extract. The resulting extract can be assayed for the capacity to antagonize CCR1

function, for example, by the assays described herein. Extracts which contain an activity that antagonizes CCR1 function can be further processed to isolate the CCR1 antagonist by suitable methods, such as, fractionation (e.g., column chromatography (e.g., ion exchange, reverse phase, affinity), phase partitioning, fractional crystallization) and assaying for biological activity (e.g., antagonism of CCR1 activity). Once isolated the structure of a natural product can be determined (e.g., by nuclear magnetic resonance (NMR)) and those of skill in the art can devise a synthetic scheme for synthesizing the natural product. Thus, a natural product can be isolated (e.g., substantially purified) from nature or can be fully or partially synthetic. A natural product can be modified (e.g., derivatized) to optimize its therapeutic potential. Thus, the term "natural product", as used herein, includes those compounds which are produced using standard medicinal chemistry techniques to optimize the therapeutic potential of a compound which can be isolated from nature.

The term "peptide", as used herein, refers to a compound consisting of from about two to about ninety amino acid residues wherein the amino group of one amino acid is linked to the carboxyl group of another amino acid by a peptide bond. A peptide can be, for example, derived or removed from a native protein by enzymatic or chemical cleavage, or can be prepared using conventional peptide synthesis techniques (e.g., solid phase synthesis) or molecular biology techniques (see Sambrook, J. *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989)). A "peptide" can comprise any suitable L- and/or D-amino acid, for example, common α-amino acids (e.g., alanine, glycine, valine), non-α-amino acids (e.g., β-alanine, 4-aminobutyric acid, 6-aminocaproic acid, sarcosine, statine), and unusual amino acids (e.g., citrulline, homocitruline, homoserine, norleucine, norvaline, ornithine). The amino, carboxyl and/or other functional groups on a peptide can be free (e.g., unmodified) or protected with a suitable protecting group. Suitable protecting groups for amino and carboxyl groups, and means for adding or removing protecting groups are know in the art and are disclosed in, for example, Green and Wuts, *"Protecting Groups in*

Organic Synthesis", John Wiley and Sons, 1991. The functional groups of a peptide can also be derivatized (e.g., alkylated) using art-known methods.

Peptides can be synthesized and assembled into libraries comprising a few to many discrete molecular species. Such libraries can be prepared using well-known methods of combinatorial chemistry, and can be screened as described herein or using other suitable methods to determine if the library comprises peptides which can antagonize CCR1 function. Such peptide antagonists can then be isolated by suitable methods.

The term "peptidomimetic", as used herein, refers to molecules which are not polypeptides, but which mimic aspects of their structures. For example, polysaccharides can be prepared that have the same functional groups as peptides which can antagonize CCR1. Peptidomimetics can be designed, for example, by establishing the three dimensional structure of a peptide agent in the environment in which it is bound or will bind to CCR1. The peptidomimetic comprises at least two components, the binding moiety or moieties and the backbone or supporting structure.

The binding moieties are the chemical atoms or groups which will react or form a complex (e.g., through hydrophobic or ionic interactions) with CCR1, for example, with the amino acid(s) at or near the ligand binding site. For example, the binding moieties in a peptidomimetic can be the same as those in a peptide antagonist of CCR1. The binding moieties can be an atom or chemical group which reacts with the receptor in the same or similar manner as the binding moiety in a peptide antagonist of CCR1. Examples of binding moieties suitable for use in designing a peptidomimetic for a basic amino acid in a peptide are nitrogen containing groups, such as amines, ammoniums, guanidines and amides or phosphoniums. Examples of binding moieties suitable for use in designing a peptidomimetic for an acidic amino acid can be, for example, carboxyl, lower alkyl carboxylic acid ester, sulfonic acid, a lower alkyl sulfonic acid ester or a phosphorous acid or ester thereof.

The supporting structure is the chemical entity that, when bound to the binding moiety or moieties, provides the three dimensional configuration of the peptidomimetic. The supporting structure can be organic or inorganic. Examples of

organic supporting structures include polysaccharides, polymers or oligomers of organic synthetic polymers (such as, polyvinyl alcohol or polylactide). It is preferred that the supporting structure possess substantially the same size and dimensions as the peptide backbone or supporting structure. This can be determined by calculating or measuring the size of the atoms and bonds of the peptide and peptidomimetic. In one embodiment, the nitrogen of the peptide bond can be substituted with oxygen or sulfur, thereby forming a polyester backbone. In another embodiment, the carbonyl can be substituted with a sulfonyl group or sulfinyl group, thereby forming a polyamide (e.g., a polysulfonamide). Reverse amides of the peptide can be made (e.g., substituting one or more -CONH- groups for a -NHCO- group). In yet another embodiment, the peptide backbone can be substituted with a polysilane backbone.

These compounds can be manufactured by known methods. For example, a polyester peptidomimetic can be prepared by substituting a hydroxyl group for the corresponding α-amino group on amino acids, thereby preparing a hydroxyacid and sequentially esterifying the hydroxyacids, optionally blocking the basic and acidic side chains to minimize side reactions. Determining an appropriate chemical synthesis route can generally be readily identified upon determining the chemical structure.

Peptidomimetics can be synthesized and assembled into libraries comprising a few to many discrete molecular species. Such libraries can be prepared using well-known methods of combinatorial chemistry, and can be screened as described herein to determine if the library comprises one or more peptidomimetics which antagonize CCR1 function. Such peptidomimetic antagonists can then be isolated by suitable methods.

In one embodiment, the CCR1 antagonist is an antibody or antigen-binding fragment thereof having specificity for CCR1. The antibody can be polyclonal or monoclonal, and the term "antibody" is intended to encompass both polyclonal and monoclonal antibodies. The terms polyclonal and monoclonal refer to the degree of homogeneity of an antibody preparation, and are not intended to be limited to particular methods of production. The term "antibody" as used herein also encompasses functional fragments of antibodies, including fragments of chimeric,

humanized, primatized, veneered or single chain antibodies. Functional fragments include antigen-binding fragments which bind to CCR1. For example, antibody fragments capable of binding to CCR1 or portions thereof, including, but not limited to Fv, Fab, Fab' and $F(ab')_2$ fragments are encompassed by the invention. Such fragments can be produced by enzymatic cleavage or by recombinant techniques. For example, papain or pepsin cleavage can generate Fab or $F(ab')_2$ fragments, respectively. Other proteases with the requisite substrate specificity can also be used to generate Fab or $F(ab')_2$ fragments. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons has been introduced upstream of the natural stop site. For example, a chimeric gene encoding a $F(ab')_2$ heavy chain portion can be designed to include DNA sequences encoding the CH_1 domain and hinge region of the heavy chain. Single chain antibodies, and chimeric, humanized or primatized (CDR-grafted), or veneered antibodies, as well as chimeric, CDR-grafted or veneered single chain antibodies, comprising portions derived from different species, and the like are also encompassed by the present invention and the term "antibody". The various portions of these antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques. For example, nucleic acids encoding a chimeric or humanized chain can be expressed to produce a contiguous protein. See, e.g., Cabilly *et al.*, U.S. Patent No. 4,816,567; Cabilly *et al.*, European Patent No. 0,125,023 B1; Boss *et al.*, U.S. Patent No. 4,816,397; Boss *et al.*, European Patent No. 0,120,694 B1; Neuberger, M.S. *et al.*, WO 86/01533; Neuberger, M.S. *et al.*, European Patent No. 0,194,276 B1; Winter, U.S. Patent No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Queen *et al.*, European Patent No. 0 451 216 B1; and Padlan, E.A. *et al.*, EP 0 519 596 A1. See also, Newman, R. *et al., BioTechnology, 10:* 1455-1460 (1992), regarding primatized antibody, and Ladner *et al.*, U.S. Patent No. 4,946,778 and Bird, R.E. *et al., Science, 242:* 423-426 (1988)) regarding single chain antibodies.

Humanized antibodies can be produced using synthetic or recombinant DNA technology using standard methods or other suitable techniques. Nucleic acid (e.g., cDNA) sequences coding for humanized variable regions can also be constructed using PCR mutagenesis methods to alter DNA sequences encoding a human or

humanized chain, such as a DNA template from a previously humanized variable region (see e.g., Kamman, M., *et al.*, *Nucl. Acids Res.*, *17*: 5404 (1989)); Sato, K., *et al.*, *Cancer Research, 53*: 851-856 (1993); Daugherty, B.L. *et al.*, *Nucleic Acids Res., 19(9):* 2471-2476 (1991); and Lewis, A.P. and J.S. Crowe, *Gene, 101*: 297-302 (1991)). Using these or other suitable methods, variants can also be readily produced. In one embodiment, cloned variable regions can be mutated, and sequences encoding variants with the desired specificity can be selected (e.g., from a phage library; see e.g., Krebber *et al.*, U.S. 5,514,548; Hoogenboom *et al.*, WO 93/06213, published April 1, 1993).

Antibodies which are specific for mammalian (e.g., human) CCR1 can be raised against an appropriate immunogen, such as isolated and/or recombinant human CCR1 or portions thereof (including synthetic molecules, such as synthetic peptides). Antibodies can also be raised by immunizing a suitable host (e.g., mouse) with cells that express CCR1, such as activated T cells (see e.g., U.S. Pat. No. 5,440,020, the entire teachings of which are incorporated herein by reference). In addition, cells expressing recombinant CCR1 such as transfected cells, can be used as immunogens or in a screen for antibody which binds receptor (See e.g., Chuntharapai *et al.*, *J. Immunol., 152:* 1783-1789 (1994); Chuntharapai *et al.*, U.S. Patent No. 5,440,021).

Preparation of immunizing antigen, and polyclonal and monoclonal antibody production can be performed using any suitable technique. A variety of methods have been described (see e.g., Kohler *et al.*, *Nature, 256:* 495-497 (1975) and *Eur. J. Immunol. 6:* 511-519 (1976); Milstein *et al.*, *Nature 266: 550-552 (1977);* Koprowski *et al.*, U.S. Patent No. 4,172,124; Harlow, E. and D. Lane, 1988, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY); *Current Protocols In Molecular Biology*, Vol. 2 (Supplement 27, Summer '94), Ausubel, F.M. *et al.*, Eds., (John Wiley & Sons: New York, NY), Chapter 11, (1991)). Generally, a hybridoma is produced by fusing a suitable immortal cell line (e.g., a myeloma cell line such as SP2/0 or P3X63Ag8.653) with antibody producing cells. The antibody producing cells, preferably those obtained from the spleen or lymph nodes, can be obtained from animals immunized with the antigen of interest. The fused cells (hybridomas) can be isolated using selective

culture conditions, and cloned by limiting dilution. Cells which produce antibodies with the desired specificity can be selected by a suitable assay (e.g., ELISA).

Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, for example, methods which select recombinant antibody from a library (e.g., a phage display library), or which rely upon immunization of transgenic animals (e.g., mice) capable of producing a repertoire of human antibodies (see e.g., Jakobovits *et al., Proc. Natl. Acad. Sci. USA, 90*: 2551-2555 (1993); Jakobovits *et al., Nature, 362*: 255-258 (1993); Lonberg *et al.*, U.S. Patent No. 5,545,806; Surani *et al.*, U.S. Patent No. 5,545,807; Lonberg *et al.*, WO97/13852).

In one embodiment, the antibody or antigen-binding fragment thereof has specificity for a mammalian CC chemokine receptor-1 (CCR1), such as human CCR1. In a preferred embodiment, the antibody or antigen-binding fragment can inhibit binding of a ligand (i.e., one or more ligands) to CCR1 and/or one or more functions mediated by CCR1 in respond to ligand binding. Preferred antibody antagonists of CCR1 function are disclosed in our co-pending United States Patent Application titled "Anti-CCR1 Antibodies and Methods of Use Therefor", by Shixin Qin, Walter Newman and Nasim Kassam, Attorney's docket number LKS97-13, U.S. Serial No. 09/239,938, filed January 29, 1999, and in International Application No. PCT/US99/04527, the teachings of each of these applications are incorporated herein by reference in their entirety.

Assessment of Activity of Antagonists

The capacity of an agent (e.g., proteins, peptides, natural products, small organic molecules, peptidomimetics) to antagonize CCR1 function can be determined using a suitable screen (e.g., high through-put assay). For example, an agent can be tested in an extracellular acidification assay, calcium flux assay, ligand binding assay or chemotaxis assay (see, for example, Hesselgesser *et al., J. Biol. Chem.* 273(25):15687-15692 (1998) and WO 98/02151).

In a particular assay, membranes can be prepared from cells which express CCR1, such as THP-1 cells (American Type Culture Collection, Manassas, VA;

Accession No. TIB202). Cells can be harvested by centrifugation, washed twice with PBS (phosphate-buffered saline), and the resulting cell pellets frozen at -70 to -85°C. The frozen pellet can be thawed in ice-cold lysis buffer consisting of 5 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid) pH 7.5, 2 mM EDTA (ethylenediaminetetraacetic acid), 5 μg/ml each aprotinin, leupeptin, and chymostatin (protease inhibitors), and 100 μg/ml PMSF (phenyl methane sulfonyl fluoride - also a protease inhibitor), at a concentration of 1 to 5 x 10^7 cells/ml, to achieve cell lysis. The resulting suspension can be mixed well to resuspend all of the frozen cell pellet. Nuclei and cell debris can be removed by centrifugation of 400 x g for 10 minutes at 4°C. The resulting supernatant can be transferred to a fresh tube and the membrane fragments can be collected by centrifugation at 25,000 x g for 30 minutes at 4°C. The resulting supernatant can be aspirated and the pellet can be resuspended in freezing buffer consisting of 10 mM HEPES pH 7.5, 300 mM sucrose, 1μg/ml each aprotinin, leupeptin, and chymostatin, and 10 μg/ml PMSF (approximately 0.1 ml per each 10^8 cells). All clumps can be resolved using a minihomogenizer, and the total protein concentration can be determined by suitable methods (e.g., Bradford assay, Lowery assay). The membrane solution can be divided into aliquots and frozen at -70 to -85°C until needed.

The membrane preparation described above can be used in a suitable binding assay. For example, membrane protein (2 to 20 μg total membrane protein) can be incubated with 0.1 to 0.2 nM ^{125}I-labeled RANTES or MIP-1α with or without unlabeled competitor (RANTES or MIP-1α) or various concentrations of compounds to be tested. ^{125}I-labeled RANTES and ^{125}I-labeled MIP-1α can be prepared by suitable methods or purchased from commercial vendors (e.g., DuPont-NEN (Boston, MA)). The binding reactions can be performed in 60 to 100 μl of a binding buffer consisting of 10 mM HEPES pH 7.2, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% BSA (bovine serum albumin), for 60 min at room temperature. The binding reactions can be terminated by harvesting the membranes by rapid filtration through glass fiber filters (e.g., GF/B or GF/C, Packard) which can be presoaked in 0.3% polyethyleneimine. The filters can be rinsed with approximately 600 μl of binding buffer containing 0.5 M NaCl, dried, and the amount of bound radioactivity can be determined by scintillation counting.

The CCR1 antagonist activity of test agents (e.g., compounds) can be reported as the inhibitor concentration required for 50% inhibition (IC_{50} values) of specific binding in receptor binding assays (e.g., using ^{125}I-RANTES or ^{125}MIP-1α as ligand and THP-1 cell membranes). Specific binding is preferably defined as the total binding (e.g., total cpm on filters) minus the non-specific binding. Non-specific binding is defined as the amount of cpm still detected in the presence of excess unlabeled competitor (e.g., RANTES or MIP-1α).

If desired, membranes prepared from cells which express recombinant CCR1 can be used in the described assay.

The capacity of compounds to antagonize CCR1 function can also be determined in a leukocyte chemotaxis assay using suitable cells. Suitable cells include, for example, cell lines, recombinant cells or isolated cells which express CCR1 and undergo CCR1 ligand-induced (e.g., MIP-1α, RANTES, MCP-2, MCP-3, MCP-4, HCC-1 or MPIF-1) chemotaxis. In one example, CCR1-expressing recombinant L1.2 cells (see Campbell, *et al. J Cell Biol, 134*:255-266 (1996)), peripheral blood mononuclear cells or HL60 cells differentiated with butyric acid, can be used in a modification of a transendothelial migration assay (Carr, M.W., *et al.* T.A., *Proc. Natl Acad Sci, USA, (91)*:3652 (1994)). Peripheral blood mononuclear cells can be isolated from whole blood by suitable methods, for example, density gradient centrifugation and positive or preferably negative selection with specific antibodies. The endothelial cells used in this assay are preferably the endothelial cell line, ECV 304, obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, U.K.). Endothelial cells can be cultured on 6.5 mm diameter Transwell culture inserts (Costar Corp., Cambridge, MA) with 3.0 μm pore size. Culture media for the ECV 304 cells can consist of M199+10% FCS, L-glutamine, and antibiotics. The assay media can consist of equal parts RPMI 1640 and M199 with 0.5% BSA. Two hours before the assay, $2x10^5$ ECV 304 cells can be plated onto each insert of the 24 well Transwell chemotaxis plate and incubated at 37°C. Chemotactic factors such as RANTES or MIP-1α (Peprotech)(diluted in assay medium) can be added to the 24-well tissue culture plates in a final volume of 600 μL. Endothelial-coated Transwells can be inserted into each well and 10^6 cells of the leukocyte type being studied are added to

the top chamber in a final volume of 100 µL of assay medium. The plate can then be incubated at 37°C in 5% CO_2/95% air for 1-2 hours. The cells that migrate to the bottom chamber during incubation can be counted, for example using flow cytometry. To count cells by flow cytometry, 500 µL of the cell suspension from the lower chamber can be placed in a tube and relative counts can be obtained for a set period of time, for example, 30 seconds. This counting method is highly reproducible and allows gating on the leukocytes and the exclusion of debris or other cell types from the analysis. Alternatively, cells can be counted with a microscope. Assays to evaluate chemotaxis inhibitors can be performed in the same way as control experiment described above, except that antagonist solutions, in assay media containing up to 1% of DMSO co-solvent, can be added to both the top and bottom chambers prior to addition of the cells. Antagonist potency can be determined by comparing the number of cell that migrate to the bottom chamber in wells which contain antagonist, to the number of cells which migrate to the bottom chamber in control wells. Control wells can contain equivalent amounts of DMSO, but no antagonist.

The activity of an antagonist of CCR1 function can also be assessed by monitoring cellular responses induced by active receptor, using suitable cells expressing receptor. For instance, exocytosis (e.g., degranulation of cells leading to release of one or more enzymes or other granule components, such as esterases (e.g., serine esterases), perforin, and/or granzymes), inflammatory mediator release (such as release of bioactive lipids such as leukotrienes (e.g., leukotriene C_4)), and respiratory burst, can be monitored by methods known in the art or other suitable methods (see e.g., Taub, D.D. *et al., J. Immunol., 155:* 3877-3888 (1995), regarding assays for release of granule-derived serine esterases; Loetscher *et al., J. Immunol., 156*: 322-327 (1996), regarding assays for enzyme and granzyme release; Rot, A. *et al., J. Exp. Med., 176*: 1489-1495 (1992) regarding respiratory burst; Bischoff, S.C. *et al., Eur. J. Immunol., 23*: 761-767 (1993) and Baggliolini, M. and C.A. Dahinden, *Immunology Today, 15*: 127-133 (1994)).

In one embodiment, an antagonist of CCR1 is identified by monitoring the release of an enzyme upon degranulation or exocytosis by a cell capable of this function. Cells expressing CCR1 can be maintained in a suitable medium under

suitable conditions, and degranulation can be induced. The cells are contacted with an agent to be tested, and enzyme release can be assessed. The release of an enzyme into the medium can be detected or measured using a suitable assay, such as in an immunological assay, or biochemical assay for enzyme activity.

The medium can be assayed directly, by introducing components of the assay (e.g., substrate, co-factors, antibody) into the medium (e.g., before, simultaneous with or after the cells and agent are combined). The assay can also be performed on medium which has been separated from the cells or further processed (e.g., fractionated) prior to assay. For example, convenient assays are available for enzymes, such as serine esterases (see e.g., Taub, D.D. *et al., J. Immunol., 155:* 3877-3888 (1995) regarding release of granule-derived serine esterases).

In another embodiment, cells expressing CCR1 are combined with a ligand of CCR1 or promoter of CCR1 function, an agent to be tested is added before, after or simultaneous therewith, and degranulation is assessed. Inhibition of ligand- or promoter-induced degranulation is indicative that the agent is an inhibitor of mammalian CCR1 function.

In a preferred embodiment, the antagonist of CCR1 function does not significantly inhibit the function of other chemokine receptors (e.g., CCR2, CXCR1, CCR3). Such CCR1-specific antagonists can be identified by suitable methods, such as by suitable modification of the methods described herein. For example, cells which do not express CCR1 ($CCR1^-$) but do express one or more other chemokine receptors (e.g., CCR2, CXCR1, CCR3) can be created or identified using suitable methods (e.g., transfection, antibody staining, western blot, RNAse protection). Such cells or cellular fractions (e.g., membranes) obtained from such cells can be used in a suitable binding assay. For example, when a cell which is $CCR1^-$ and $CCR3^+$ is chosen, the CCR1 antagonist can be assayed for the capacity to inhibit the binding of a suitable CCR3 ligand (e.g., RANTES, MCP-3) to the cell or cellular fraction, as described herein.

In another preferred embodiment, the antagonist of CCR1 function is an agent which binds to CCR1. Such CCR1-binding antagonists can be identified by suitable methods, for example, in binding assays employing a labeled (e.g., enzymatically

labeled (e.g., alkaline phosphatase, horse radish peroxidase), biotinylated, radio-labeled (e.g., ^{3}H, ^{14}C, ^{125}I)) antagonist.

In another preferred embodiment, the antagonist of CCR1 function is an agent which can inhibit the binding of a (i.e., one or more) CCR1 ligand to CCR1 (e.g., human CCR1).

In particularly preferred embodiment, the antagonist of CCR1 function is an agent which can bind to CCR1 and thereby inhibit the binding of a (i.e., one or more) CCR1 ligand to CCR1 (e.g., human CCR1).

Methods of Therapy

The term "graft", as used herein, refers to organs and/or tissues which can be obtained from a first mammal or donor and transplanted into a second mammal, preferably a human. The term "graft" encompasses, for example, skin, eye or portions of the eye (e.g., cornea, retina, lens), muscle, bone marrow or cellular components of the bone marrow (e.g., stem cells, progenitor cells), heart, lung, heart-lung (e.g., heart and a single lung, heart and both lungs), liver, kidney, pancreas, parathyroid, bowel (e.g., colon, small intestine, duodenum), neuronal tissue, bone and vasculature (e.g., artery, vein). A graft can be obtain from a suitable mammal (e.g., human, pig, baboon, chimpanzee), or under certain circumstances a graft can be produced *in vitro* by culturing cells, for example, embryonal cells, fetal cells, skin cells, blood cells and bone marrow cells which were obtained from a suitable mammal. The graft can be obtained from a genetically modified animal or can be modified (e.g., genetically, chemically, physically) by suitable means. A graft is preferably obtained from a human. An "allograft", as the term is used herein, refers to a graft comprising antigens which are allelic variants of the corresponding antigens found in the recipient. For example, a human graft comprising an MHC class II antigen encoded by the HLA-DRB1*0401 allele is an allograft if transplanted into a human recipient whose genome does not comprise the HLA-DRB1*0401 allele.

In one embodiment, the method of inhibiting (reducing or preventing) graft rejection comprises administering an effective amount of an (i.e., one or more) antagonist of CCR1 function to a recipient of a graft. In another embodiment, the

method of inhibiting graft rejection comprises administering an effective amount of an antagonist of CCR1 function to a recipient of an allograft. In a preferred embodiment, the method comprises administering an effective amount of an antagonist of CCR1 function to a recipient of a cardiac allograft.

In another embodiment, the antagonist of CCR1 function is selected from the group consisting of small organic molecules, natural products, peptides, peptidomimetics and proteins, wherein said proteins are not chemokines or mutants or analogues thereof.

In a preferred embodiment, the invention provides a method for inhibiting (reducing or preventing) graft rejection comprising administering to a graft recipient an effective amount of an antagonist of CCR1 function and an effective amount of an (i.e., one or more) additional therapeutic agent, preferably, an immunosuppressive agent. Advantageously, the rejection-inhibiting effects of CCR1 antagonists and immunosuppressive agents can be additive or synergistic, and can result in permanent engraftment.

A further benefit of co-administration of a CCR1 antagonist and an immunosuppressive agent is that the dose of immunosuppressive agent required to inhibit graft rejection can be reduced to sub-therapeutic levels (e.g., a dose that does not inhibit graft rejection when administered as the sole therapeutic agent). The ability to reduce the dose of the immunosuppressive agent can greatly benefit the graft recipient as many immunosuppressive agents have severe and well-known side effects including, for example, increased incidence of infection, increased incidence of certain malignancies, diabetes mellitus, neurotoxicity, nephrotoxicity, hyperlipidemia, hypertension, hirsutism, gingival hyperplasia, impaired wound healing, lymphopenia, jaundice, anemia, alopecia and thrombocytopenia (Spencer, C.M., *et al.*, *Drugs*, *54*(6):925-975 (1997); *Physicians Desk Reference*, 53rd Edition, Medical Economics Co., pp. 2081-2082 (1999)).

The term "immunosuppressive agent", as used herein, refers to compounds which can inhibit an immune response. The immunosuppressive agent used in the invention can be a novel compound or can be selected from the compounds which are known in the art, for example, calcineurin inhibitors (e.g., cyclosporin A,

FK-506), IL-2 signal transduction inhibitors (e.g., rapamycin), glucocorticoids (e.g., prednisone, dexamethosone, methylprednisolone), nucleic acid synthesis inhibitors (e.g., azathioprine, mercaptopurine, mycophenolic acid) and antibodies to lymphocytes or antigen-binding fragments thereof (e.g., OKT3, anti-IL2 receptor). Novel immunosuppressive agents can be identified by those of skill in the art by suitable methods, for example, screening compounds for the capacity to inhibit antigen-dependent T cell activation.

The immunosuppressive agent used for co-therapy (e.g., co-administration with an antagonist of CCR1 function) is preferably a calcineurin inhibitor. More preferably the immunosuppressive agent used for co-therapy is cyclosporin A.

When the graft is bone marrow, cells (e.g., leukocytes) derived from the graft can mount an immune response directed at the recipient's organs and tissues. Such a condition is referred to in the art as graft versus host disease (GVHD). Administration of an antagonist of CCR1 function with or without an additional therapeutic agent (e.g., immunosuppressive agent, hematopoietic growth factor) can inhibit GVHD. Accordingly, in another embodiment, the invention provides a method of inhibiting (reducing or preventing) GVHD in a bone marrow graft recipient comprising administering an effective amount of an antagonist of CCR1 function. In an additional embodiment, the method of inhibiting GVHD comprises the administration of an antagonist of CCR1 function and one or more additional therapeutic agents, for example, an immunosuppressive agent.

In another embodiment, the method of inhibiting GVHD comprises the administration of an antagonist of CCR1 function, which is selected from the group consisting of small organic molecules, natural products, peptides, peptidomimetics and proteins, wherein said proteins are not chemokines or mutants or analogues thereof.

A further aspect of the invention related to inhibiting ischemia/reperfusion injury using CCR1 antagonists. Ischemia/reperfusion injury refers to necrotic cell death that occurs when the flow of blood to an organ or tissue is restricted or stopped (ischemia), resulting in oxygen deprivation (hypoxia). The injury sustained by an organ or tissue under ischemic conditions is apparent after blood flow has been restored (reperfusion). Ischemia/reperfusion injury can be the result of a pathological

condition in which ischemia can occur, for example, myocardial infarction, arteriosclerosis, stroke, transient ischemic attacks and the like. Ischemia/reperfusion injury can also result from trauma or medical procedures which stop, restrict, or redirect (e.g., shunt) the flow of blood. Examples of such trauma include, for example, frost bite, burns and pinch injuries which restrict the flow of blood to the limbs or extremities. Medical procedures which can result in ischemia/reperfusion injury include, for example, placement of a tourniquet, angioplasty (e.g., balloon angioplasty) and surgery (e.g., organ transplantation). In the context of organ transplantation, ischemia/reperfusion injury which occurs during the transplantation of all grafts, even isogenic grafts (e.g., when the donor and recipient are identical twins), can be a contributing factor to graft rejection (e.g., acute rejection, chronic rejection).

A murine model of cold renal ischemia/reperfusion injury, which mimics the storage conditions of organs (e.g., kidneys) that have been removed from a donor in preparation for transplantation, is disclosed herein (see Example 3). Studies using this model revealed that inhibiting CCR1 function can significantly inhibit ischemia/reperfusion injury. Specifically, renal ischemia/reperfusion had no measurable effect on the renal function of CCR1 KO mice with 100% of the mice surviving for the 72 hour follow-up period. In contrast, CCR1 +/+ control mice experienced considerable impairment of renal function and 80% mortality at 72 hours (Table 3). Histological examination of kidneys removed for the CCR1 KO and CCR1 +/+ control mice 48 hours after ischemia/reperfusion revealed that inhibiting CCR1 function can inhibit tubular necrosis and the infiltration of neutrophils into the kidney. Thus, by inhibiting CCR1 function ischemia/reperfusion injury can be inhibited (reduced or prevented).

Accordingly, disruption of CCR1 function can have significant beneficial actions in preventing injury to transplanted grafts (e.g., kidneys). For example, inhibiting CCR1 function can inhibit (reduce or prevent) initial post-transplant graft injury, and thereby, lead to reduced acute and chronic allograft rejection. Furthermore, inhibiting CCR1 function can enlarge the organ donor pool since many organs (e.g., kidneys) are not transplanted because they are thought to be at high risk of ischemia/reperfusion injury as a result of prolonged storage.

Accordingly, another embodiment of the invention is a method for inhibiting ischemia/reperfusion injury comprising administering to a subject (e.g., a human) in need thereof an effective amount of an antagonist of CCR1 function. In certain embodiments, the ischemia/reperfusion injury can be a consequence of a medical procedure, trauma or a result of a pathological condition. In a particular embodiment, the invention provides a method for inhibiting ischemia/reperfusion injury which can occur during graft transplantation. In a preferred embodiment, the invention provides a method for inhibiting ischemia/reperfusion injury which can occur during transplantation of a kidney.

In another embodiment, the method of inhibiting ischemia/reperfusion injury comprises the administration of an antagonist of CCR1 function, which is selected from the group consisting of small organic molecules, natural products, peptides, peptidomimetics and proteins, wherein said proteins are not chemokines or mutants or analogues thereof.

In another embodiment, the method of inhibiting ischemia/reperfusion injury comprises administering to a subject in need thereof an effective amount of an antagonist of CCR1 function and an effective amount of one or more additional therapeutic agents which can promote blood flow and/or inhibit leukocyte infiltration. For example, the additional agent can be selected from the group consisting of, a fibrinolytic agent (e.g., Retavase), a thrombolytic agent, such as a plasminogen activator (e.g., tissue plasminogen activator, urokinase, streptokinase, recombinant tissue plasminogen activator), an anticoagulant (e.g., a coumarin anticoagulant (e.g., warfarin, ethyledine dicoumarol), heparin, hirulog, hirudin, aspirin), a vasodilator (e.g., nitroglycerin, amotriphene, erythritol, prenylamine), an agent which stimulates or inhibits the production of nitric oxide (e.g., a stimulator or inhibitor of nitric oxide synthase, for example, the compounds disclosed in U.S. Patent Nos.: 5,811,437 issued to Singh *et al.*, 5,854,234 issued to Hansen *et al.* and 5,854,251 issued to Hallinan *et al.*), an immunosuppressive agent and a cell adhesion inhibitor. Cell adhesion inhibitors suitable for co-administration with an antagonists of CCR1 function include, for example, proteins, such as, cytokines, antibodies which bind to cell adhesion molecules (e.g., integrins, selectins) and antigen-binding fragments thereof, and soluble adhesion molecules (e.g., chimeric adhesion

molecules), small organic molecules, peptides and peptidomimetics, for example, the compounds disclosed in United States Patent Nos: 5,843, 441 issued to Gundel *et al.*, 5,695,760 issued to Faanes *et al.*, 5,843,425 issued to Tedder *et al.*, 5,753,617 and 5,710,123, issued to Heavner *et al.*, 5,837,689 issued to Anderson *et al.*, 5,725,802 issued to Barrett *et al.*, 5,510,332 issued to Kogan *et al.*, 5,707,985 and 5,260,277 issued to McKenzie *et al.*, International Patent Applications WO 97/03094, WO 98/04247, WO 96/22966 by Biogen Inc., WO 97/10839, WO 96/00581 by Texas Biotechnology Corporation, WO 94/15958, WO 93/08823, WO 92/08464 by Tanabe Seiyaku Co. Ltd. The entire teaching of each of the above cited references in incorporated herein by reference.

In a preferred embodiment, the additional agent that is co-administered with the antagonist of CCR1 function can be selected from the group consisting of an immunosuppressive agent and a cell adhesion inhibitor. In another preferred embodiment, the additional agent can be selected from the group consisting of a fibrinolytic agent, a thrombolytic agent, an anti-coagulant, a vasodilator and an agent which stimulates or inhibits the production of nitric oxide.

The invention further relates to an antagonist of CCR1 function for use in therapy (including prophylaxis), for example, as described herein, and to the use of such an antagonist for the manufacture of a medicament for inhibiting graft rejection (e.g., acute rejection, chronic rejection) and/or ischemia/reperfusion injury as described herein. The invention also relates to a medicament for inhibiting graft rejection (e.g., acute rejection, chronic rejection) and/or ischemia/reperfusion injury wherein said medicament comprises an antagonist of CCR1 function.

A "subject" is preferably a human, but can also be a mammal in need of veterinary treatment, e.g., domestic animals (e.g., dogs, cats, and the like), farm animals (e.g., cows, sheep, fowl, pigs, horses, and the like) and laboratory animals (e.g., rats, mice, guinea pigs, and the like).

An effective amount of the antagonist of CCR1 function can be administered to a subject to inhibit (reduce or prevent) graft rejection and/or ischemia/reperfusion injury. For example, an effective amount of the antagonist of CCR1 function can be administered before, during and/or after transplant surgery or other medical procedure which can result in ischemia/reperfusion injury.

When co-administration of an antagonist of CCR1 function and an additional therapeutic agent is indicated or desired for inhibiting graft rejection and/or ischemia/reperfusion injury, the antagonist of CCR1 function can be administered before, concurrently with or after administration of the additional therapeutic agent. When the antagonist of CCR1 function and additional therapeutic agent are administered at different times, they are preferably administered within a suitable time period to provide substantial overlap of the pharmacological activity (e.g., inhibition of CCR1 function, immunosuppression) of the agents. The skilled artisan will be able to determine the appropriate timing for co-administration of an antagonist of CCR1 function and an additional therapeutic agent depending on the particular agents selected and other factors.

An "effective amount" of a CCR1 antagonist is an amount sufficient to achieve a desired therapeutic and/or prophylactic effect, such as an amount sufficient to inhibit inhibit graft rejectiona and/or ischemia/reperfusion injury. For Example, an effective amount is an amount sufficient to inhibit a (i.e., one or more) function of CCR1 (e.g., CCR1 ligand-induced leukocyte migration, CCR1 ligand-induced integrin activation, CCR1 ligand-induced transient increase in the concentration of intracellular free calcium $[Ca^{2+}]_i$ and/or CCR1 ligand-induced secretion (e.g. degranulation) of proinflammatory mediators), and thereby, inhibit graft rejectiona and/or ischemia/reperfusion injury. An "effective amount" of an additional therapeutic agent (e.g., immunosuppressive agent) is an amount sufficient to achieve a desired therapeutic and/or prophylactic effect (e.g., immunosuppression).

The amount of agent (e.g., CCR1 antagonist, additional therapeutic agent) administered to the individual will depend on the characteristics of the individual, such as general health, age, sex, body weight and tolerance to drugs as well as the degree, severity and type of rejection and/or ischemia/reperfusion injury. The skilled artisan will be able to determine appropriate dosages depending on these and other factors. Typically, an effective amount can range from about 0.1 mg per day to about 100 mg per day for an adult. Preferably, the dosage ranges from about 1 mg per day to about 100 mg per day.

The agent (e.g., CCR1 antagonist, additional therapeutic agent) can be administered by any suitable route, including, for example, orally in capsules,

suspensions or tablets or by parenteral administration. Parenteral administration can include, for example, intramuscular, intravenous, subcutaneous, or intraperitoneal administration. The agent (e.g., CCR1 antagonist, additional therapeutic agent) can also be administered orally (e.g., dietary), transdermally, topically, by inhalation (e.g., intrabronchial, intranasal, oral inhalation or intranasal drops) or rectally. Administration can be local or systemic as indicated. The preferred mode of administration can vary depending upon the particular agent (e.g., CCR1 antagonist, additional therapeutic agent) chosen, however, oral or parenteral administration is generally preferred.

The agent (e.g., CCR1 antagonist, additional therapeutic agent) can be administered as a neutral compound or as a salt. Salts of compounds containing an amine or other basic group can be obtained, for example, by reacting with a suitable organic or inorganic acid, such as hydrogen chloride, hydrogen bromide, acetic acid, perchloric acid and the like. Compounds with a quaternary ammonium group also contain a counteranion such as chloride, bromide, iodide, acetate, perchlorate and the like. Salts of compounds containing a carboxylic acid or other acidic functional group can be prepared by reacting with a suitable base, for example, a hydroxide base. Salts of acidic functional groups contain a countercation such as sodium, potassium and the like.

The antagonist of CCR1 function can be administered to the individual as part of a pharmaceutical composition for inhibition of graft rejection and/or ischemia/reperfusion injury comprising a CCR1 antagonist and a pharmaceutically acceptable carrier. Pharmaceutical compositions for co-therapy can comprise an antagonist of CCR1 function and one or more additional therapeutic agents. An antagonist of CCR1 function and an additional therapeutic agent can be components of separate pharmaceutical compositions which can be mixed together prior to administration or administered separately. Formulation will vary according to the route of administration selected (e.g., solution, emulsion, capsule). Suitable pharmaceutical carriers can contain inert ingredients which do not interact with the antagonist of CCR1 function and/or additional therapeutic agent. Standard pharmaceutical formulation techniques can be employed, such as those described in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA.

Suitable pharmaceutical carriers for parenteral administration include, for example, sterile water, physiological saline, bacteriostatic saline (saline containing about 0.9% mg/ml benzyl alcohol), phosphate-buffered saline, Hank's solution, Ringer's-lactate and the like. Methods for encapsulating compositions (such as in a coating of hard gelatin or cyclodextran) are known in the art (Baker, *et al.*, "Controlled Release of Biological Active Agents", John Wiley and Sons, 1986).

The present invention will now be illustrated by the following Examples, which are not intended to be limiting in any way.

EXAMPLES

Example 1 CCR1 Targeting and Cardiac Transplantation

Experimental design

3 sets of data were generated:

(I) The tempo of acute rejection was determined in CCR1 knockout (KO) vs. control mice.

(ii) The effect of immunosuppression with cyclosporin A (CsA) on CCR1 KO vs. control mice was determined.

(iii) The effects of CCR1 deletion on development of chronic rejection was determined.

Methods

Mice. CCR1 KO mice (also refered to as CCR1 -/-)(B6/129 strain, H-2^b), which are homozygous for a target gene disruption of CCR1, were produced by Craig Gerard (Children's Hospital, Boston, MA; Gerard, C. *et al.*, *J. Clin. Invest. 100*:2022-2027 (1997)) and were bred at LeukoSite (Cambridge, Massachusetts). All other mice were obtained from Jackson Laboratory (Bar Harbor, ME). These included donor strains (BALB/c, H-2^d; B6.C-H2(bm12)/KhEg(bm12), H-2^b) and control recipients (B6/129). BALB/c and B6/129 differ at both class I and class II major histocompatibility complex (MHC) loci, whereas Bm12 and B6/129 differ at MHC class II alone.

Mouse cardiac allografting (Mottram, P.L. *et al.*, *Transplantation 59*:559-565 (1995); Hancock, W.W., *et al.*, *Proc. Natl. Acad. Sci (USA), 93*:13967-13972 (1996)) was performed with the aid of an operating microscope (Nikon, 4x to 38x magnification) under clean conditions (rigorously sterile conditions were not required for studies involving CCR1 KO or control mice, whereas they are required for use of other immunodeficient mice):

Preparation of the donor heart. Donor mice were anesthetized with Nembutal (50 mg/10 g body weight) and Atropine sulfate (0.17 mg/100 g body weight) i.p.; additional anaesthesia with Methoxyflurane supplementation was administered via a face mask as required during the procedure. Mice were shaved and cleansed with 70% alcohol. A midline abdominal incision was made in the donor animal and 1 ml of a 10% solution of heparin in saline was injected into the inferior vena cava. The incision was then extended cephalic to open the chest through a median sternotomy. The thorax was opened. The inferior vena cava was ligated with 6-0 silk and divided inferior to the tie. The superior vena cava was then similarly ligated and divided superior to the tie. The aorta and pulmonary artery were separated and divided as far distally as possible. At this point, blood was evacuated from the heart by applying pressure with applicator sticks. The aorta was transected just proximal to the brachiocephalic artery and the main pulmonary artery transected just proximal to its bifurcation. The pulmonary veins were then ligated and divided en mass and the heart placed in iced saline.

Preparation of the recipient. After being anesthetized in the same way as the donor, the recipient was brought under the microscope, a midline abdominal incision was made, and segments of the aorta and vena cava below the renal vessels were dissected free, but not separated from each other, over a length of about 2 mm. A clamp was placed on the proximal aorta and vena cava, and a distal tie of 6-0 silk was placed around both the aorta and vena cava in preparation for later occlusion of the vessels.

Transplantation of the heart. The tie that had been placed around the distal aorta and vena cava was secured by means of a single knot. An aortotomy and a venotomy in the vena cava were made adjacent to one another. The donor heart was then removed from the chilled saline, and the donor aorta and pulmonary artery were

joined end-to-side to the recipient aorta and vena cava, respectively, with running suture, using 10-0 tipped with a BV-3 needle. Since the anastomoses were done adjacent to one another, the side of the pulmonary artery-cava suture line next to the aortic anastomosis was sutured from the inside with an everting running suture. During this period, chilled saline was dripped on the ischemic heart at frequent intervals. After the completion of the anastomoses, the inferior vascular occluding tie was released first, thus filling the inferior vena cava and donor pulmonary artery with recipient venous blood. Upon release of the proximal occluding tie, the aorta and coronary arteries of the transplant were perfused with oxygenated recipient blood. Blood loss was minimized by gradual release of the proximal tie. Warm saline was used externally to warm the heart immediately after establishing coronary perfusion. With warming and coronary perfusion, the heart began to fibrillate and usually within a few minutes it reverted spontaneously to a sinus rhythm. Occasionally, cardiac massage was required to re-establish a normal beat. The intestines were placed carefully back into the abdominal cavity around the auxiliary heart, and the abdomen was closed with a single running suture to all layers (saline with antibiotic was used to wash the peritoneal cavity as needed). The mouse was then placed in a constant temperature at 35°C for recovery from anesthesia.

Immunosuppression. The effect of cyclosporin A (CsA) (Sigma, St. Louis, MO) therapy (10 mg/kg/day, injected subcutaneously) was tested by using our standard cardiac allograft model of BALB/c → B6/129 or B6/129→CCR1 KO and injecting CsA daily until rejection or for a maximum of 21 days, beginning on the day of transplantation.

Monitoring of allograft survival. Cardiac allograft survival was monitored twice daily by palpation of ventricular contractions through the abdominal wall (Mottram, P.L. *et al.*, *Transplantation, 59*:559-565 (1995)), rejection was defined as the day of cessation of palpable heartbeat, and was verified by autopsy (Gerard, C, *et al., J. Clin Invest., 100:*2022-2027 (1997); Mottram, P.L.,*et al., Transplantation, 59:*559-565 (1995)). Once cardiac graft function ceased, mice were anesthetized as above, and grafts were surgically excised, subdivided into portions for (a) formalin fixation, paraffin embedding and subsequent light microscopy examination, or (b)

snap-frozen in liquid nitrogen and stored at -70°C until processed for immunohistology or RNAse protection assays.

Immunopathology. For histology, paraffin sections were stained with hematoxylin and eosin (H&E) to evaluate graft morphology, and with Weigert's elastin stain so as to examine the extent of intimal proliferation in penetrating branches of myocardial arteries (a key feature of transplant arterioscelerosis). (Gerard, C, *et al., J. Clin Invest. 100:*2022-2027 (1997); Mottram, P.L., *et al., Transplantation 59:*559-565 (1995)). Chemokine and chemokine receptor mRNA expression was determined using RNAse protection assay kits (Pharmingen, San Diego, CA).

Results

Allograft survival data (mean ± SD) are summarized in Table 1 (using 6-10 animals/group)

Table 1 - Effect of CCR1 KO on mouse cardiac allograft survival

#	Strains (Donor → Recipient)	Therapy (mismatch)	Survival (days)	p value
1	Balb/C → B6/129	nil (class I & II)	7.7±0.8	
2	Balb/C → CCR1 KO	nil (class I & II)	12.9±0.7	p<0.0001 vs #1
3	Balb/C → B6/129	low dose CsA, 3 wk	10.3±0.8	
4	Balb/C → CCR1 KO	low dose CsA, 3 wk	>100	p<0.005 vs #3
5	Bm12 → B6/129	nil (class II only)	34.0±1.3	
6	Bm12 → CCR1 KO	nil (class II only)	>100	p<0.005 vs #5

p values were determined by the Mann-Whitney U test.

The key points arising from Table 1 are:

$CCR1^+$ cells contribute to the pathogenesis of allograft rejection.

Disruption of CCR1 function in a complete MHC mismatch significantly prolongs allograft rejection (group 1 vs. 2). As anticipated, rejection in control mice was associated with graft infiltration by $CCR1^+$ mononuclear cells (mainly

macrophages), and mRNA studies showed that rejection in control mice was associated with intragraft induction of expression of the mRNAs for the CCR1 ligands, MIP-1α and RANTES. Though rejection in CCR1 KO mice was accompanied by dense mononuclear cell infiltration and upregulation of CCR1 ligand mRNA, allografts lacked CCR1 mRNA or protein expression.

Addition of CsA (10 mg/kg/d) caused only a minor prolongation of allograft survival in control mice (2-3 days) as compared with untreated recipients (group 1 vs. 3). However, the same dose of CsA in CCR1 KO mice (for a maximum of 21 days) led to permanent engraftment in all recipients (group 4, further evaluation revealed that mice of group 4 survived for >200 days following cardiac transplant). The beneficial actions of some experimental agents can be undermined by concomitant immunosuppression. However, CsA and inhibition of CCR1 function are synergistic in efficacy. In addition, grafts harvested at day 100 from CCR1 KO recipients showed only a minor mononuclear cell infiltrate and no evidence of transplant arteriosclerosis. These findings are in contrast to the severe arteriosclerosis observed in control allograft recipients which were treated with high dose CsA (30 mg/kg/d) or CD4 mAb therapy (Mottram, P.L., Han, W. R., *et al.*, "Increased expression of IL-4 and IL-10 and decreased expression of IL-2 and IFN-γ in long-surviving mouse heart allografts after brief CD4-monoclonal antibody therapy," *Transplantation 59:*559-565 (1995); Hancock, W.W., Buelow, R., *et al.*, "Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes," *Nature Medicine 4:* 1392-1396 (1998)).

Disruption of CCR1 function in class II-mismatch combinations is highly efficacious. Whereas untreated recipients rejected their allografts at around day 35 (group 5), disruption of CCR1 function in this combination resulted in permanent engraftment (group 6). Moreover, as with group 4 (low dose CsA), allografts harvested at day 100 showed no evidence of transplant arteriosclerosis or other features of chronic rejection.

Example 2 CCR1 and Chronic Rejection in Cardiac Allograft Recipients

Administration of CD4 monoclonal antibody (mAb) can prolong the survival of cardiac allografts in the described murine model (Mottram *et al.*, *Transplantation*

59:559-565 (1995)). However, the extended survival of grafts in anti-CD4 treated animals is complicated by the development of chronic rejection with florid transplant arteriosclerosis (Hancock *et al.*, *Nature Medicine 4*:1392-1396 (1998)).

Methods

Cardiac allografts derived from Balb/c donors were transplanted into CCR1 KO or CCR1 +/+ control mice as described in Example 1.

Immunosuppression. CD4 mAb (GK1.5, American Type Culture Collection, Manassas, VA; Accession No. TIB-207) was administered four times to CCR1 -/- or CCR1 +/+ allograft recipients (6/group); 250 μg by intraperitoneal injection on day 0 (time of transplantation) and on subsequent days 1, 2 and 3.

Monitoring of chronic rejection. Cardiac allograft survival was monitored twice daily by palpation of ventricular contractions through the abdominal wall. All grafts survived to day 60, and the readout was morphologic examination, particularly, the extent of development of transplant arteriosclerosis. Accordingly, grafts were fixed in formalin, embedded in paraffin and sections counterstained with Weigert's elastin stain. All intramyocardial arteries were scored for the extent of intimal proliferation as <5% occlusion (0), >5-20% (1), >20-40% (2), >40-60% (3), >60-80% (4) or >80-100% (5) (Murphy *et al.*, *Transplantation 64*:14-19 (1997)).

Results

Results of scoring of vessels within cardiac allografts (6 grafts/group) and statistical evaluation (Mann-Whitney U test) are presented in Table 2.

Table 2 - Effect of CCR1 KO on development of transplant arteriosclerosis

#	Strains (Donor → Recipient)	Total Vessels	Vessel Score (mean ± SD)	p value
1	Balb/c → B6/129	23	2.4 ± 1.1	
2	Balb/c → CCR1 KO	31	0.3 ± 0.2	p<0.0001 vs. #1

The key points arising from Table 2 are:

$CCR1^+$ cells contribute to the pathogenesis of chronic allograft rejection.

Disruption of CCR1 function blocks the development of transplant atherosclerosis.

Disruption of CCR1 function blocks the development of other features of chronic rejection.

Example 3 CCR1 and Renal Ischemia/Reperfusion Injury

Prolonged interruption of blood supply to an organ will lead to its complete necrosis even if the circulation is eventually restored. Interruption for shorter periods can lead to death of specific cell types which are highly susceptible to hypoxia but the overall organ can recover to a partial or even complete extent depending upon the period of lack of blood flow (ischemia) and other factors. The damage, known as ischemia/reperfusion injury, which is apparent following restoration of blood flow to a transiently ischemic kidney or other commonly transplanted organs is primarily mediated by neutrophils, which are recruited to the organ upon revascularization. Though multiple pathways are implicated in such neutrophil recruitment, we hypothesized that the chemokine receptor, CCR1, might play a key role such that blockade of this pathway would ameliorate injury following ischemia/reperfusion. We established a new mouse model of cold renal ischemia/reperfusion injury in which maintaining the organ at 4°C during the period of ischemia mimics the storage conditions of a kidney which has been harvested in preparation for transplantation. Hence, these observations relate directly to ways in which clinical ischemia/reperfusion injury in the context or renal allografting might be decreased, or even eliminated, by blockade of the CCR1 pathway.

Experimental design

Kidneys of inbred CCR1 KO mice or control B6/129 were perfused *in situ* until pale, using cold saline, and packed in ice for varying periods prior to revascularization. After preliminary studies, we undertook 60 minutes of cold ischemia and followed-up for 48-72 hours, with analysis of renal function, histology, immunopathology (with quantitative image analysis) and mRNA analysis.

Methods

Mice. CCR1 KO mice (B6/129 strain, H-2^b) were produced by Craig Gerard (Children's Hospital, Boston, MA; Gerard, C. *et al.*, *J. Clin. Invest. 100*:2022-2027 (1997)) and were bred at LeukoSite (Cambridge, Massachusetts). Control B6/129 mice were obtained from Jackson Laboratory (Bar Harbor, ME).

Ischemia/reperfusion model. The ischemia/reperfusion procedure was performed under sterile conditions with the aid of an operating microscope (Zeiss, 2-50x magnifications). Mice (CCR1 KO or B6/129) were anesthetized with Nembutal (50 mg/100 g body weight); Methoxyflurane supplementation, administered via a facemask, was used to provide additional anesthesia, as required. After shaving and disinfecting the abdomen with 70% alcohol, the laparotomy was performed via a midline incision. After access to the right kidney's renal artery and vein, the suprarenal and infrarenal vena cava and aorta were separated, the peri-renal tissue was cut and the kidney was mobilized. To stop arterial bloodflow to the kidney infra- and suprarenal aorta were closed by application of microclips. An aortomy was then performed and the kidney was flushed with cold saline, until pale. To enable the backflow of the flushing solution in the abdomen, a small incision was performed at the renal vein, between clip and kidney after clamping the vein with a microclip. All incisions were sutured with 10-0 prolene sutures. After this procedure the kidneys were exposed for varying ischemic times of up to 75 minutes, throughout which times the kidneys were kept cold by packing them in ice.

After removing the clips and reperfusion of the organ, a contralateral nephrectomy was performed. The abdomen was closed with 6-0 silk. The animals were kept post-operatively in a heated cage, under observation, until they were seen to have recovered from the procedure.

Monitoring of the effects of ischemia/reperfusion injury. Serum creatinine levels were measured daily. Since creatinine levels peaked at 48 hours post-ischemia/reperfusion, mice (8/group) were either sacrificed at 48 hours for histologic and mRNA studies of the kidneys, or were followed for 72 hours to determine effects on animal survival. For histology, kidneys were fixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) and periodic

acid/Schiff (PAS) stain. Chemokine and chemokine receptor mRNA expression were determined using RNAse protection assay kits (Pharmingen, San Diego, CA).

Results

The contrasting findings in control B6/129 vs. CCR1 mice are shown in Table 3.

Table 3 - Effect of CCR1 KO on mouse renal ischemia/reperfusion injury

Group (n=8)	Creatinine (60 minutes , 48 hrs)	Tubules	Neutrophil infiltration	Follow-up (72 hrs)
B6/129	1.9±1.1	25-50% tubular necrosis	dense peri-tubular aggregates	20% survival
CCR1 KO	0.4±0.1	<5% necrosis	absent	100% survival
CCR1 KO vs. B6/129	p<0.005	p<0.001	p<0.001	p<0.05

p values were determined by the Mann-Whitney U test.

The key points arising from Table 3 are:

Disruption of CCR1 function blocked neutrophil recruitment and completely prevented the impairment of renal function following ischemia/reperfusion injury. CCR1 blockade also almost completely suppressed tubular cell necrosis.

Not shown in Table 3 are the results of mRNA analysis. Kidneys from both CCR1 KO and B6/129 mice showed induction of the expression of mRNA for the CCR1-associated chemokines, MIP-1α and RANTES. Thus, the effects of CCR1 deletion left pro-inflammatory cell responses intact, but the absence of CCR1 on host cells inhibited leukocyte recruitment and subsequent renal injury.

The study was expanded by increasing the number of animals in each group to 15, and extending the analysis period to 100 days. The results reported in Table 3 were included in the analysis of the expanded study. The expanded study

demonstrated that CCR1-/- mice survived significantly longer after ischemia/reperfusion than B6/129 mice (92 ± 6.1 vs. 45 ± 12.4 days, mean survival time ± standard error of the mean, limited to 100 days, $p<0.001$). 86% of CCR1-/- mice were alive 100 days following the ischemia/reperfusion procedure, while only 26% of B6/129 mice survived for 100 days following the procedure (Figure 1). Measurements of serum creatinine were made 2, 7 and 100 days after the ischemia/reperfusion procedure to assess renal function. CCR1-/- mice were protected from renal injury and showed serum creatinine levels which were similar to those detected in sham animals (unilateral nephrectomy no ischemia/reperfusion). However, serum creatinine levels were significantly increased, by about 5-fold, in wild type B6/129 animals two days after ischemia/reperfusion ($p<0.005$). Significantly elevated (4-fold) serum creatinine levels were also detected in wild type B6/129 animals seven days after ischemia/reperfusion ($p<0.005$). Serum creatinine was elevated in all groups, including sham animals, 100 days after the procedure ($p<0.01$)(Figure 2).

Example 4 CCR1 and Hepatic Ischemia/Reperfusion Injury

Methods

Male C57B6/129 (B6/129) mice or male CCR1-/- mice weighing 22-28 g were used. Partial hepatic ischemia was induced in mice anesthetized with sodium pentobarbital (60 mg/kg i.p.). A midline laparotomy was performed and an atraumatic clip was used to interrupt the blood supply to the middle and left lobes of the liver, thereby producing partial hepatic ischemia. After 90 minutes the clip was removed, initiating hepatic reperfusion. Sham control mice underwent the same surgical procedure without interruption of the blood supply to the middle and left lobes of the liver. Mice were sacrificed at serial intervals and hepatic tissue and blood samples were taken for analysis. Liver function was assessed by measuring serum alanine aminotransferase (SGPT) and/or serum aspartate aminotransferase (SGOT).

Results

CCR1-/- mice were protected against liver dysfunction over 0.5-7 days following hepatic ischemia/reperfusion, as assessed by serum alanine aminotransferase (SGPT) (Figure 3) and serum aspartate aminotransferase (SGOT) assays. In addition, CCR1-/- mice survived significantly longer than B6/129 wild type mice following hepatic ischemia/reperfusion (13.4 ± 3.6 days compared to 8.7 ± 5.1 days, $p<0.01$). Immunohistological analysis of hepatic tissue demonstrated ischemia/reperfusion-induced expression of CCR1 ligands in CCR1-/- and B6/129 mice. Widespread hepatic necrosis and neutrophil infiltration was observed in liver tissue removed from B6/129 mice following hepatic ischemia/reperfusion. However, only minor and focal hepatocyte injury and no neutrophil infiltration was seen in hepatic tissue of CCR1-/- mice after hepatic ischemia/reperfusion. Thus, the absence of CCR1 in mice inhibited hepatic ischemia/reperfusion injury and subsequent liver dysfunction.

Example 5 Immunocompetence of CCR1-/- mice

In Vitro T cell Proliferative Responses

Mixed lymphocyte responses (MLR) were assessed by culturing responder splenocytes (isolated from CCR1-/- or wild type B6/129 mice) with mitomycin-C inactivated allogeneic stimulator splenocytes (isolated from Balb/c mice) in RPMI-1640 medium containing 5% FBS, 1% penicillin/streptomycin and 5×10^{-5} M 2-mercaptoethanol, in 96 well flat-bottom plates. Cultures were incubated at 37°C in 5% CO_2 for 3 to 5 days and were pulsed with [^{3}H]thymidine for 6 hours before harvesting. The amount of [^{3}H]thymidine incorporated by the cultured cells was measured by scintillation counting. The mean amount of radioactivity incorporated (counts per minute) and standard deviations were calculated using 12 wells per group.

Mitogen-induced proliferation of T cells was measured using Concanavilin-A (Con-A, a T cell mitogen). Splenocytes isolated from CCR1-/- or wild type B6/129 mice were cultured in 96 well flat-bottom plates in RPMI-1640 medium containing 5% FBS, 1% penicillin/streptomycin, 5×10^{-5} M 2-mercaptoethanol and 1.25-10 µg/mL Con-A (Sigma Chemical Co., St. Louis, MO). The cultures were incubated at

37°C in 5% CO_2 for 72 hours and were pulsed with [^{3}H]thymidine for 6 hours before harvesting. The amount of [^{3}H]thymidine incorporated by the cultured cells was measured by scintillation counting. The mean amount of radioactivity incorporated (counts per minute) and standard deviation were calculated using 12 wells per group.

Results

The results of the in vitro T cell proliferation studies are presented in Figures 4A and 4B. The mitogen-induced T cell proliferation detected in cultures of splenocytes from CCR1-/- mice was identical to that detected in cultures of splenocytes from wild type B6/129 mice (Figure 4A). CCR1-/- mice developed a vigorous response to allogeneic stimulator cells in MLR assays ($p<0.001$) (Figure 4B). However, the overall magnitude of the response in repeated 5-day MLR assays was consistently 20-25% lower in cultures containing responder splenocytes isolated from CCR1 -/- mice in comparison to cultures containing B6/129 responder splenocytes ($p<0.01$). These studies of *in vitro* T cell responses demonstrate that CCR1-/- mice are immunocompetent.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details can be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

CLAIMS

What is claimed is:

1. A method of inhibiting graft rejection comprising administering to a subject in need thereof an effective amount of an antagonist of CCR1 function.

2. The method of Claim 1 wherein said graft is an allograft.

3. The method of Claim 2 wherein said allograft is selected from the group consisting of kidney, liver, lung, heart-lung, pancreas, bowel and heart.

4. The method of Claim 3 wherein said allograft is a heart.

5. The method of Claim 1 wherein said antagonist of CCR1 function is selected from the group consisting of small organic molecules, natural products, peptides, proteins and peptidomimetics.

6. The method of Claim 5 wherein said antagonist of CCR1 function is a small organic molecule.

7. The method of Claim 5 wherein said antagonist of CCR1 function is a natural product.

8. The method of Claim 5 wherein said antagonist of CCR1 function is a peptide.

9. The method of Claim 5 wherein said antagonist of CCR1 function is a peptidomimetic.

10. The method of Claim 5 wherein said antagonist of CCR1 function is a protein.

11. The method of Claim 10 wherein said protein is an anti-CCR1 antibody or antigen-binding fragment thereof.

12. A method of inhibiting graft rejection comprising administering to a subject in need thereof an effective amount of an antagonist of CCR1 function and an immunosuppressive agent.

13. The method of Claim 12 wherein said immunosuppressive agent is one or more agents selected from the group consisting of calcineurin inhibitors, glucocorticoids, nucleic acid synthesis inhibitors, and antibodies which bind to lymphocytes or antigen-binding fragments thereof.

14. The method of Claim 13 wherein said immunosuppressive agent is a calcineurin inhibitor.

15. The method of Claim 14 wherein said calcineurin inhibitor is cyclosporin A.

16. The method of Claim 14 wherein said calcineurin inhibitor is FK-506.

17. The method of Claim 13 wherein said immunosuppressive agent is a glucocorticoid.

18. The method of Claim 17 wherein said glucocorticoid is prednisone or methylprednisolone.

19. A method of inhibiting graft versus host disease comprising administering an effective amount of an antagonist of CCR1 function to a recipient of a transplanted graft.

20. The method of Claim 19 wherein said graft is bone marrow.

21. The method of Claim 20 further comprising administering an immunosuppressive agent.

22. The method of Claim 21 wherein said immunosuppressive agent is a calcineurin inhibitor.

23. The method of Claim 22 wherein said calcineurin inhibitor is cyclosporin A or FK-506.

24. A method of inhibiting ischemia/reperfusion injury comprising administering to a subject in need thereof an effective amount of an antagonist of CCR1 function.

25. The method of Claim 24 wherein said antagonist of CCR1 function is selected from the group consisting of small organic molecules, natural products, peptides, proteins and peptidomimetics.

26. The method of Claim 25 wherein said antagonist of CCR1 function is a small organic molecule.

27. The method of Claim 25 wherein said antagonist of CCR1 function is a natural product.

28. The method of Claim 25 wherein said antagonist of CCR1 function is a peptide.

29. The method of Claim 25 wherein said antagonist of CCR1 function is a peptidomimetic.

30. The method of Claim 25 wherein said antagonist of CCR1 function is a protein.

31. The method of Claim 30 wherein said protein is an anti-CCR1 antibody or antigen-binding fragment thereof.

32. The method of Claim 25 wherein said ischemia/reperfusion injury is a consequence of a medical procedure which stops, restricts or redirects the flow of blood.

33. The method of Claim 32 wherein said medical procedure is angioplasty or surgery.

34. The method of Claim 32 wherein said medical procedure is surgery.

35. The method of Claim 34 wherein said surgery is graft transplantation.

36. The method of Claim 35 wherein said graft is selected from the group consisting of kidney, lung, liver, heart-lung, pancreas, bowel and heart.

37. The method of Claim 36 wherein said graft is a kidney.

38. The method of Claim 24 wherein said ischemia/reperfusion injury is the result of a pathological condition selected from the group consisting of arteriosclerosis, myocardial infarction, stroke and transient ischemic attack.

39. The method of Claim 38 wherein said pathological condition is myocardial infarction.

40. The method of Claim 38 wherein said pathological condition is stroke.

41. The method of Claim 38 wherein said pathological condition is arteriosclerosis.

42. The method of Claim 24 further comprising administering to said subject an effective amount of one or more additional therapeutic agents selected from the group consisting of fibrinolytic agents, thrombolytic agents, anti-coagulants, cell adhesion inhibitors, anti-thrombotic agents, stimulators of nitric oxide synthase and inhibitors of nitric oxide synthase.

43. The method of Claim 42 wherein said additional therapeutic agent is a fibrinolytic agent.

44. The method of Claim 42 wherein said additional therapeutic agent is a thrombolytic agent.

45. The method of Claim 42 wherein said additional therapeutic agent is an anti-coagulant.

46. The method of Claim 42 wherein said additional therapeutic agent is a cell adhesion inhibitor.

47. The method of Claim 42 wherein said additional therapeutic agent is an anti-thrombotic agent.

48. The method of Claim 42 wherein said additional therapeutic agent is an activator or inhibitor of nitric oxide synthase.

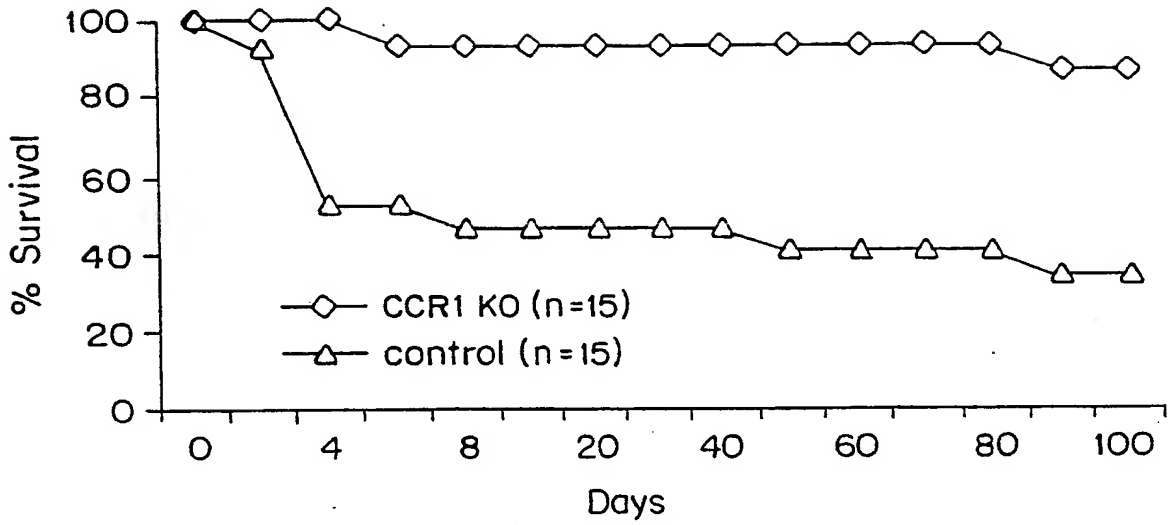

FIG. 1

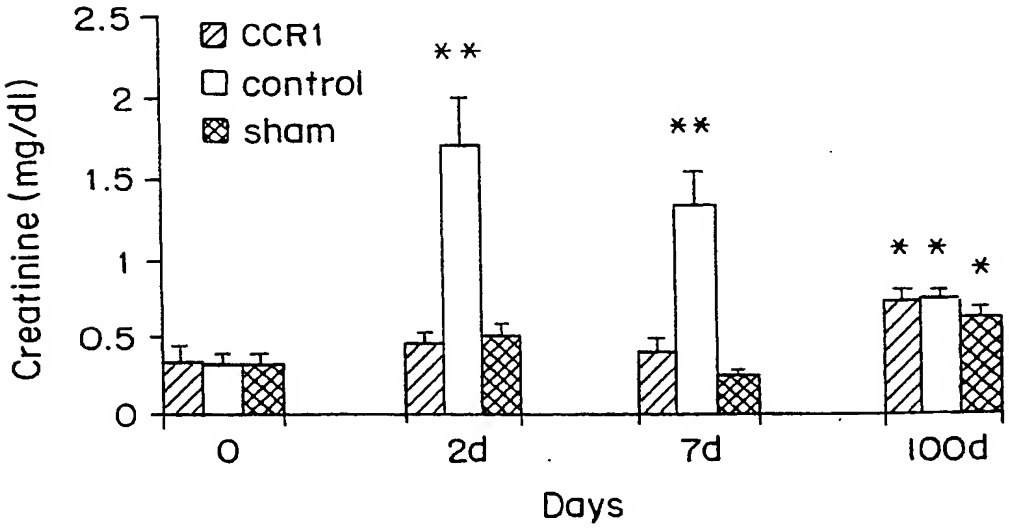

FIG. 2

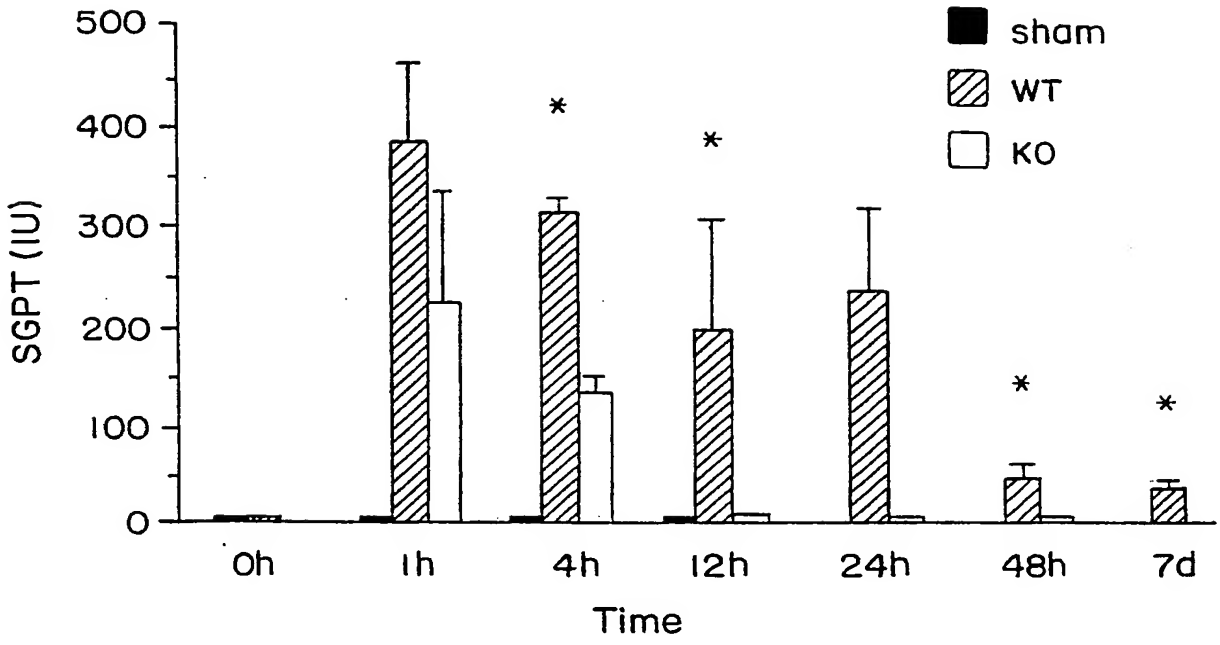

FIG. 3

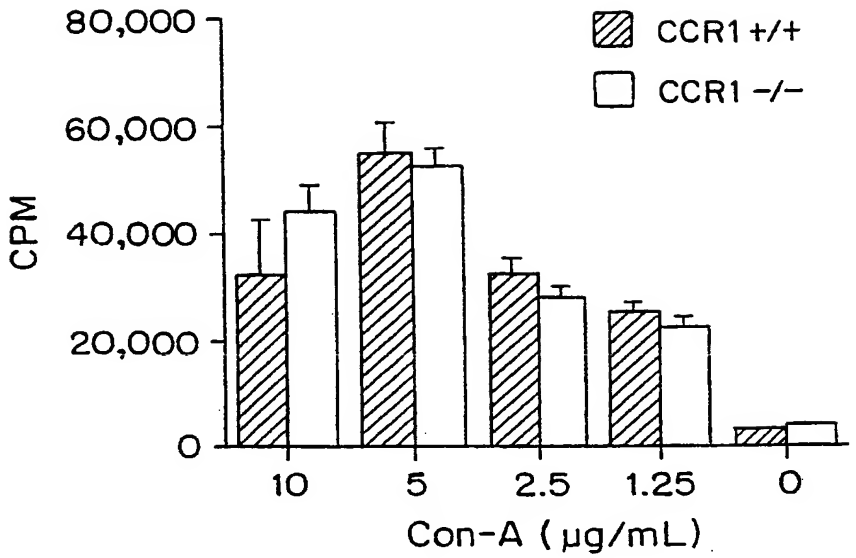

FIG. 4A

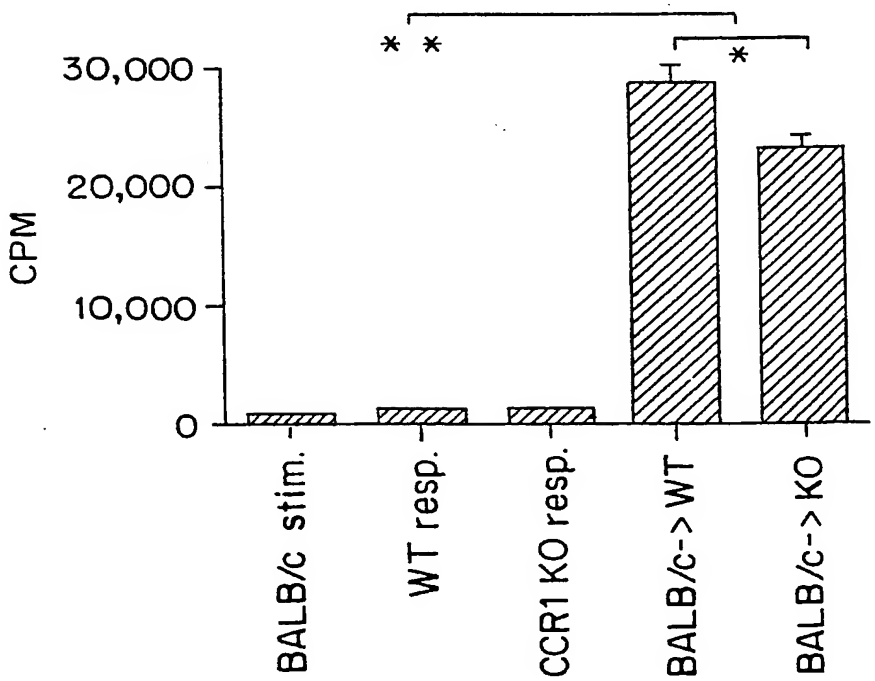

FIG. 4B

INTERNATIONAL SEARCH REPORT

In .ational Application No
PCT/US 00/02123

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/00 C07K16/28 C07K14/52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 99 40061 A (PFIZER PRODUCTS INC.) 12 August 1999 (1999-08-12) page 1, line 9 - line 22 page 29, line 13 - line 19 claims 1-7	1-6, 12-26, 32-48

X,P	WO 99 37619 A (LEUKOSITE INC.) 29 July 1999 (1999-07-29) cited in the application page 7, line 12 - line 27 claims 1-46	1-6, 12-26, 32-48

X,P	WO 99 37617 A (LEUKOSITE INC.) 29 July 1999 (1999-07-29) cited in the application claims 1-25 page 6, line 12 - line 18	1-6, 12-26, 32-48

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 June 2000	26/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Siatou, E

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/02123

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 27815 A (MERCK & CO INC) 2 July 1998 (1998-07-02) cited in the application claims 1-6 page 16, line 32 -page 17, line 30 page 20, line 12 -page 22, line 29	1-6, 12-26, 32-48
X	WO 98 38167 A (PFIZER INC.) 3 September 1998 (1998-09-03) cited in the application claims 1-68 page 1, line 9 - line 22	1-6, 12-26, 32-48
X	WO 98 31364 A (MERCK & CO. INC.) 23 July 1998 (1998-07-23) cited in the application page 18, line 16 - line 19 page 20, line 31 -page 21, line 21 claims 10-18	1-6, 12-26, 32-48
X	WO 98 09642 A (THE UNITED STATES OF AMERICA) 12 March 1998 (1998-03-12) cited in the application claims 1-14 page 5, line 37 -page 6, line 3 page 7, line 31 - line 35	1-5,8, 10, 12-25, 28,30, 32-48
X	WO 96 38559 A (DANA FARBER CANCER INSTITUTE) 5 December 1996 (1996-12-05) claims 1-11 page 11, line 29 -page 12, line 3 page 12, line 12 - line 25	1-5,8, 10,19, 20,24, 25,28, 30,32-41
A	J. HESSELGESSER ET AL: "Identification and Characterisation of Small Molecule Functional Antagonists of the CCR1 Chemokine Receptor" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 25, 19 June 1998 (1998-06-19), pages 15687-15692, XP002139458 cited in the application	1-48

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INTERNATIONAL SEARCH REPORT

In. ational Application No
PCT/US 00/02123

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	O. M. Z. HOWARD ET AL: "Inhibition of in Vitro and in Vivo HIV Replication by a Distamycin Analogue that Interferes with Chemokine Receptor Function: A Candidate for Chemotherapeutic and Microbicidal Application" JOURNAL OF MEDICINAL CHEMISTRY, vol. 41, no. 13, 1998, pages 2184-2193, XP002139459 cited in the application ---	1-48
A	SU S B ET AL: "Preparation of specific polyclonal antibodies to a C-C chemokine receptor, CCR1, and determination of CCR1 expression of various types of leukocytes" JOURNAL OF LEUKOCYTE BIOLOGY,US,FEDERATION OF AMERICAN SOCIETIES FOR EXPERIMENTAL, vol. 60, no. 5, November 1996 (1996-11), pages 658-666-666, XP002123620 ISSN: 0741-5400 cited in the application ---	1-48
A	C. GERARD ET AL: "Targeted Disruption of the beta-Chemokine Receptor CCR1 Protects against Pancreatitis -associated Lung Injury" JOURNAL OF CLINICAL INVESTIGATIONS, vol. 100, 1997, pages 2022-2027, XP002139460 cited in the application abstract -----	1-48

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-48 relate to methodfor inhibiting gaft rejection or reperfusion injury using compounds defined by reference to a desirable characteristic or property, namely antagonism of CCR1 function. The claims cover all methods using compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such methods. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the method by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the use of compounds mentioned in the description page 8, line 14-page 9, line 11 for inhibiting graft rejection or reperfusion injury.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ational Application No
PCT/US 00/02123

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9940061 A	12-08-1999	AU 1778999 A	23-08-1999
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		EP 0828833 A	18-03-1998
		JP 11506005 T	02-06-1999
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